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Identification of cytosolic phosphodiesterases in the erythrocyte: A possible role for PDE5

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Within erythrocytes (RBCs), cAMP levels are regulated by phosphodiesterases (PDEs). Increases in cAMP and ATP release associated with activation of β-adrenergic receptors (βARs) and prostacyclin receptors (IPRs) are regulated by PDEs 2, 4 and PDE 3, respectively. Here we establish the presence of cytosolic PDEs in RBCs and determine a role for PDE5 in regulating levels of cGMP.

Material/Methods: Purified cytosolic proteins were obtained from isolated human RBCs and western analysis was performed using antibodies against PDEs 3A, 4 and 5. Rabbit RBCs were incubated with dbcGMP, a cGMP analog, to determine the effect of cGMP on cAMP levels. To determine if cGMP affects receptor-mediated increases in cAMP, rabbit RBCs were incubated with dbcGMP prior to addition of isoproterenol (ISO), a βAR receptor agonist. To demonstrate that endogenous cGMP produces the same effect, rabbit and human RBCs were incubated with SpNONOate (SpNO), a nitric oxide donor, and YC1, a direct activator of soluble guanylyl cyclase (sGC), in the absence and presence of a selective PDE5 inhibitor, zaprinast (ZAP).

Results: Western analysis identified PDEs 3A, 4D and 5A. dbcGMP produced a concentration dependent increase in cAMP and ISO-induced increases in cAMP were potentiated by dbcGMP. In addition, incubation with YC1 and SpNO in the presence of ZAP potentiated βAR-induced increases in cAMP.

Conclusions: PDEs 2, 3A and 5 are present in the cytosol of human RBCs. PDE5 activity in RBCs regulates cGMP levels. Increases in intracellular cGMP augment cAMP levels. These studies suggest a novel role for PDE5 in erythrocytes.

key words: **red blood cell • cGMP • isoproterenol • PDE5 • zaprinast**

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Background

Activation of G-protein coupled receptors present on mammalian erythrocytes stimulates the synthesis of cAMP, a cyclic nucleotide involved in signaling pathways that culminate in the release of ATP [1]. It has been demonstrated that, in human and rabbit erythrocytes, receptor-mediated activation of either the prostacyclin receptor (IPR) or β-adrenergic receptor (βAR) stimulates adenylyl cyclase (AC) activity resulting in increases in cAMP and subsequently ATP release [1,2]. ATP is a stimulus for the synthesis and release of endothelium-derived relaxing factors [3,4]. The regulated release of ATP allows the erythrocyte to participate in the local control of vascular caliber [3,5,6].

The magnitude and duration of increases in cAMP, as well as the localization of those increases to discrete signaling pathways, requires local control of the rates of cAMP synthesis by AC and its hydrolysis by phosphodiesterases (PDEs) [7–9]. PDE enzymes include 11 families that differ in their genetic derivation, molecular structure, substrate specificity, inhibitor sensitivity and mode of regulation [10,11]. PDEs are the sole known physiological means of inactivation of cyclic nucleotide activity in cells. It has become clear that specific PDEs are associated with individual signaling pathways. Importantly, this specificity permits increases in cAMP to be compartmentalized, allowing activation of individual receptors to produce discrete cellular responses [7,12,13].

The activity of several PDEs that hydrolyze cAMP has been demonstrated in both human and rabbit erythrocytes [14–16]. In addition, the presence of PDE 2A and 3B protein has been identified in membranes of these cells [14,16]. It has been shown that PDE3, a cGMP-inhibited PDE, regulates cAMP increases associated with activation of the IPR [14–16] while PDE2, a cGMP-activated PDE, as well as PDE4 are associated with regulation of increases in cAMP resulting from activation of the βAR in erythrocytes [15]. Here we demonstrate the presence of two PDE isoforms that hydrolyze cAMP in the cytosol of human erythrocytes, PDEs 3A and 4D. We also report for the first time that PDE5, a PDE that hydrolyzes cGMP is present in the cytosol of these cells. In addition, we examined the contribution of increases in cGMP and the activity of PDE5 to the regulation of cAMP levels under basal conditions as well as in response to receptor-mediated activation of the βAR. Finally, we show that cGMP and PDE5 can participate in the regulation of cAMP levels in these cells.

Material and Methods

Isolation of erythrocytes

Male New Zealand white rabbits were anesthetized with ketamine (12.5 mg/kg) and xylazine (1.5 mg/kg) intramuscularly, followed by pentobarbital sodium (10 mg/kg) administered via a cannula placed in an ear vein. A catheter was subsequently placed in a carotid artery and heparin (500 units) was administered. After 10 min, the animals were exsanguinated. Human blood was obtained by venipuncture using a syringe containing heparin (500 units). Immediately after collection of blood, erythrocytes were isolated by centrifugation at 500 × g for 10 min at 4°C with the supernatant and buffy coat removed by aspiration. Packed erythrocytes were re-suspended and washed 3 times in a physiological salt solution containing (in mM); 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21.0 Tris-base and 5.5 dextrose with 0.5% bovine serum albumin, pH adjusted to 7.4. Erythrocytes were prepared on the day of use. The protocols for blood collection from rabbits and humans were approved by the Institutional Animal Care and Use Committee and the Institutional Review Board of Saint Louis University, respectively.

Isolation of protein from erythrocyte cytosol

Isolated packed human erythrocytes, 3 ml, were lysed in ice cold hypotonic buffer (5mM NaPi, pH 7.5/ 0.5mM EGTA) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche) and centrifuged at 30,000 × g for 20 min to separate the cytosolic fraction from the membrane fraction. The supernatant (cytosolic fraction) was removed and the pellet (membrane fraction) was discarded. To remove hemoglobin from the cytosol, a DE52 column was equilibrated with binding buffer (200 mM Tris/HCl [pH 7.5], 200mM NaCl, and 5 mM EGTA) diluted ten-fold with H_2O and supplemented with 5 mM $MgCl_2$ and 1 mM DTT. The column was packed to 3–4 cm in height. The cytosol was loaded onto the column (6 ml/1 ml DE52 matrix) and cleared of hemoglobin with 3 washes of 1mL of binding buffer. The remaining cytosolic bound proteins were eluted with elution buffer containing (3 ml of 0.4M NaCl) in binding buffer. The eluate was dialyzed overnight in 1L of buffer containing (in mM); 21.0 tris(hydroxymethyl)aminomethane, 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl, 1.2 $MgSO_4$) and concentrated on Centricon-10 spin concentrator (Amicon) to a volume of 200–250 µl [17].

Western analysis

Purified cytosolic proteins were solubilized in SDS buffer (0.277 M SDS, 60% glycerol, 0.25 M Tris-HCl (pH 6.8), 0.004% bromophenol blue, and 0.400 M dithiothreitol). The sample was boiled and 40 µg of protein was loaded onto a pre-cast gel (Pierce) and subjected to electrophoresis. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were blocked overnight with 5% non-fat dry milk in PBS containing 0.1% Tween-20. PDE4 isoforms were identified by immunoblotting with an affinity purified PDE4D antibody directed against the C-terminus of all PDE4D variants (Fabgennix). To identify PDE3A, PVDF membranes were blocked overnight with starting block (Thermo Scientific) containing 0.05% Tween-20 then immunoblotted with two different goat polyclonal antibodies directed against either the N-or C-terminus of PDE3A (Santa Cruz). Finally, PDE5A isoforms were identified by immunoblotting with an affinity purified PDE5A antibody generated against peptides from unique sequences of the PDE5A gene (Fabgennix). The PVDF membranes were then incubated with an appropriate secondary antibody in 1% non-fat dry milk or starting block and protein-antibody complexes were visualized using enhanced chemiluminesence (Pierce). These selected antibodies have been used in other studies to identify the resepective PDEs [18–22].

Effect of cGMP on basal and receptor-mediated increases in cAMP in rabbit erythrocytes

Isolated rabbit erythrocytes were diluted to a 50% hematocrit (1 ml) and incubated with increasing concentrations of the cGMP analog, dbcGMP (1, 10 or 30 μM, Biomol) or its vehicle, saline for 30 min prior to determination of cAMP. In separate studies, rabbit erythrocytes were incubated absence and presence of dbcGMP (10 μM) prior to stimulation with the β-agonist, isoproterenol (ISO, 1 μM) for 30 min in order to determine the effect of cGMP on receptor-mediated increases in cAMP. Importantly, the cGMP analog does not interfere with EIA determination of cAMP levels (GE Healthcare).

Effect of activators of soluble guanylyl cyclase (sGC) and a PDE5 inhibitor on basal and ISO-induced increases in cAMP in rabbit and human erythrocytes

Isolated rabbit and human erythrocytes (50% hematocrit) were pretreated with YC1 (sGC activator, 100 μM) and the nitric oxide donor spermine nonoate (SpNO, 100 nM) in the presence and absence of either the selective PDE5 inhibitor, zaprinast (ZAP, 10 μM) or the PDE1 inhibitor, vinpocetine (VIN, 30 μM) for 30 min prior to addition of ISO (1 μM) for determination of cAMP. The vehicles for the agonists used were saline (ISO, SpNO) or N',N-dimethylformamide (DMF) (YC1, ZAP, VIN). The vehicles had no effect on basal cAMP levels. The concentrations of zaprinast and vinpocetine were chosen based on published IC_{50} values for inhibition of PDE5 in other cell types and our previous experience with VIN [1,14,23,24]. At the concentrations chosen the inhibition of the respective PDE is highly selective [10,25–29].

Measurement of cAMP

Reactions were stopped with the addition of 4 ml ice-cold acidified ethanol containing 1 mM HCl per 1 ml of erythrocyte suspension. The erythrocyte-ethanol mixture was centrifuged at 14,000 × g for 10 min at 4°C, to remove precipitated proteins. The supernatant was removed and stored overnight at –20°C. Samples were centrifuged a second time at 3,700 × g for 10 min at 4°C, to remove cryoprecipitates. The supernatant was again removed and dried under vacuum centrifugation. Concentrations of cAMP were determined by EIA (GE Healthcare) according to the manufacturer's instructions. Cell counts were obtained from the erythrocyte suspension prior to addition of acidified ethanol and cAMP values were corrected to 1×10^{10} erythrocytes/ml.

Measurement of cGMP

Samples were prepared as described above for cAMP determination and concentrations of cGMP were determined by EIA acetylation procedure (GE Healthcare). Cell counts were obtained from the erythrocyte suspension prior to addition of acifided ethanol and cAMP values were corrected to 1×10^{10} erythrocytes/ml.

Data analysis

Statistical significance was determined using an analysis of variance (ANOVA). In the event that the *F*-ratio indicated a change had occurred, a Fisher's LSD test was performed to identify individual differences. Results are reported as means ± the standard error of the mean (SEM).

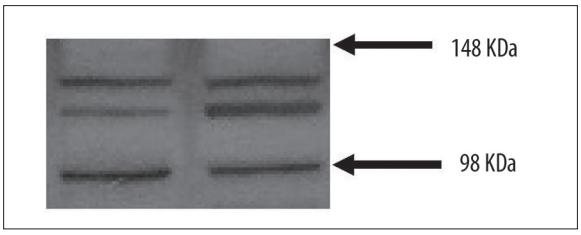

Figure 1. Identification of PDE4D isoforms in human erythrocytes. Erythrocyte cytosol preparations were incubated with an affinity purified primary antibody generated against the C-terminus of all PDE4D variants (representative of 5 individual samples, 40 μg protein per lane).

Results

Identification of PDE4 in the cytosol of human erythrocytes

PDE2 and PDE4 activity regulate increases in cAMP stimulated by isoproterenol (ISO)-induced activation of the βAR. PDE2 protein is present in human erythrocyte membranes [14]. In other cell types, it has been demonstrated that PDE4D splice variants 3, 4 or 5 are associated with this receptor [13,30,31]. Using Western analysis, we determined that PDE4D is present in human erythrocyte cytosol preparations. A non-isoform selective PDE4D antibody identified three bands with predicted molecular weights of 119, 105 and 98 KDa corresponding to those of PDE4D4, 5 and 3, respectively (Figure 1), [17,32].

Identification of PDE3A in the cytosol of human erythrocytes

PDE3B protein is present in human erythrocyte membranes and inhibitors of PDE3 activity augment increases in cAMP produced by activation of the prostacyclin receptor (IPR) in these cells [14–16]. However, PDE3 inhibitors inactivate both isoforms of PDE3, PDE3A and PDE3B, and it is the 3A isoform that has been associated with IPR signaling in human platelets [33,34]. There are three splice variants of PDE3A (3A1, A2, and A3) [35–37]. Using antibodies generated against both the N-(Figure 2A) and C-terminus (Figure 2B) of human PDE3A, we identified a band with a predicted molecular weight of 74 KDa which corresponds to the molecular weight reported for the PDE3A3 isoform [36,38]

Identification of PDE5 in the cytosol of human erythrocytes

Activation of the particulate or soluble form of guanylyl cyclase increases cGMP in human erythrocytes [39,40]. However, the contribution of PDEs to the regulation of cGMP levels in erythrocytes has not been fully characterized. Here, cytosol preparations were probed with an antibody against the N-terminus of human PDE5A. This antibody identified a band with a predicted molecular weight of 105 KDa which corresponds to that of PDE5A (Figure 3) [27,41–43].

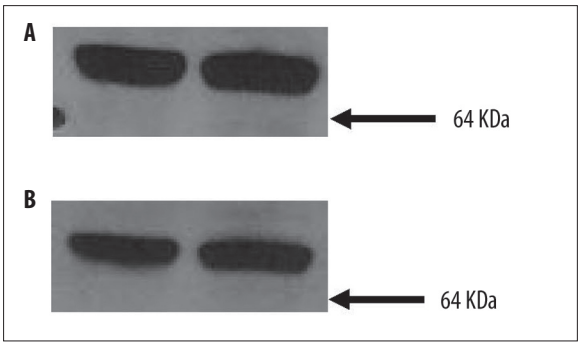

Figure 2. (**A**) Identification of the PDE3A isoform in human erythrocytes. Erythrocyte cytosol preparations were incubated with a goat polyclonal primary antibody directed against the N-terminus of PDE3A (representative of 8 individual samples, 40 µg protein per lane). (**B**) Identification of the PDE3A isoform in human erythrocytes. Erythrocyte cytosol preparations were incubated with a goat polyclonal primary antibody against the C-terminus of PDE3A (representative of 8 individual samples, 40 µg protein per lane).

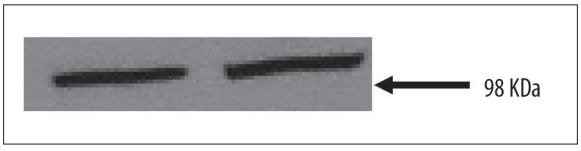

Figure 3. Identification of the PDE5A isoform in human erythrocytes. Erythrocyte cytosol preparations were incubated with an affinity purified primary antibody against the C-terminus of PDE5A (representative of 5 individual samples, 40 µg protein per lane).

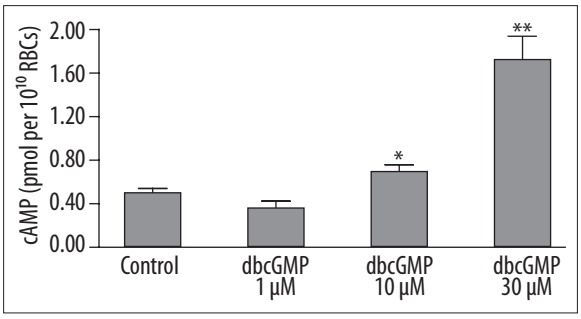

Figure 4. Effect of the dbcGMP (10 µM) on cAMP levels in rabbit erythrocytes (n=4). Erythrocytes were incubated with dbcGMP for 30 min. Values are means ±SE. * different from control (N',N-dimethylformamide, DMF), ($p<0.01$); ** different from all other values ($P<0.01$).

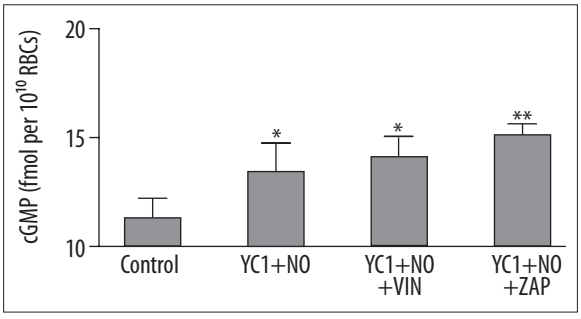

Figure 5. Effect of YC1 (100 µM) & spermine NONOate (NO, 100 nM) or the combination of YC1 and NO in the presence of either zaprinast (ZAP, 10 µM) or vinpocetine (VIN, 30 µM) on cGMP levels in rabbit erythrocytes (n=4). Erythrocytes were incubated with either ZAP or VIN for 30 min prior to stimulation with YC1 and spermine NONOate for 20 min. Values are means ±SE. * different from control (N',N-dimethylformamide, DMF), ($p<0.01$); ** different from all other values ($P<0.05$).

Effect of a cGMP analog on cAMP levels in erythrocytes

It has been reported that cGMP can inhibit PDE3 and activate PDEs 2 and 5 [11,22]. To determine the effect of cGMP on cAMP levels in rabbit erythrocytes, we incubated these cells with a cell permeable cGMP analog, dbcGMP that is hydrolyzed to cGMP in erythrocytes [40,44]. Incubation of rabbit erythrocytes with dbcGMP (1 to 30 µM) resulted in concentration-dependent increases in cAMP (Figure 4).

Effect of an activator of soluble guanylyl cyclase (sGC), YC1, a nitric oxide donor, spermine NONOate (SpNO) and selective inhibitors of either PDE5, zaprinast (ZAP), or PDE1, vinpocetine (VIN), on cGMP levels in rabbit erythrocytes

Since stimulation of sGC has been reported to generate increases in cGMP in human erythrocytes, we determined if: 1) stimulation of sGC with YC1 and SpNO would generate increases in cGMP in these cells and if 2) inhibitors of cGMP-hydrolysing PDEs would potentiate that increase. Rabbit erythrocytes were incubated with YC1 (100 µM) and SpNO (100 nM) in the presence and absence of either VIN (30 µM) or ZAP (10 µM). Stimulation of sGC increased cGMP in these cells (Figure 5). The increases were potentiated by the selective PDE5 inhibitor, ZAP whereas, the selective PDE1 inhibitor, VIN had no effect on cGMP increases (Figure 5). At the concentration chosen neither VIN nor ZAP has an effect on baseline cGMP levels (data not shown).

Effect of cGMP on ISO-induced increases in cAMP in rabbit and human erythrocytes

In non-erythroid cells, cGMP has been shown to either positively or negatively influence increases in cAMP produced by activation of the βAR depending on the profile of PDEs present [23,45]. To determine the effect of cGMP on ISO-induced increases in cAMP in erythrocytes, cells were incubated with ISO (1 µM) in the absence and presence of dbcGMP (10 µM). The addition of dbcGMP potentiated ISO-induced increases in cAMP in rabbit erythrocytes (Figure 6). To determine if increases in endogenous cGMP synthesis would have the same effect, erythrocytes were incubated with the combination of (SpNO, 100 nM) and YC1 (100 µM) in the presence and absence of VIN (30 µM) or ZAP (10 µM) prior to addition of ISO (1 µM). The combination of activation of sGC and inhibition of PDE5 (ZAP) potentiated ISO-induced increases in cAMP in both rabbit (Figure 7A) and human erythrocytes (Figure 7B), while inhibition of PDE1 had no effect.

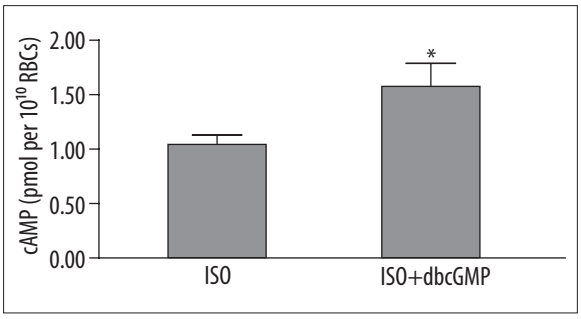

Figure 6. Effect of the dbcGMP (10 μM) on isoproterenol (ISO, 1 μM)-induced increases in cAMP levels in rabbit erythrocytes (n=9). Erythrocytes were incubated with dbcGMP for 30 min prior to stimulation with ISO for 30 min. Values are means ±SE. * different from control (saline), ($p<0.01$).

Discussion

Cyclic nucleotides are multifunctional second messenger molecules that mediate diverse physiological functions in cells [22,46]. To prevent cAMP or cGMP generated in one signaling pathway from activating unwanted downstream effectors of another pathway or to control interaction between pathways, local levels of these cyclic nucleotides must be precisely regulated. It has been increasingly recognized that such regulation is accomplished by PDEs that are localized to individual signaling pathways. Indeed, it has been suggested that, rather than total expression of PDEs, it is the compartmentalization of specific PDEs that is the important factor in modulating localized cAMP levels [9,29]. Here we report, for the first time, the presence of three cytosolic PDEs in human erythrocytes and suggest a role for cGMP and PDE5 in the regulation of cAMP levels in these cells.

Previously, using selective inhibitors, it was demonstrated that the activity of PDE4 and PDE3 regulates cAMP levels associated with activation of the β-adrenergic receptor (βAR) and prostacyclin receptor (IPR), respectively [14]. Here, using Western analysis, we determined that the PDE4D isoform known to be associated with the βAR pathway [47,48] as well as PDE3A, the PDE3 isoform associated with hydrolysis of IPR-mediated increases in cAMP in platelets [33], are present in erythrocytes (Figures 1, 2). We also demonstrated the presence of PDE5, a cGMP-specific PDE in the cytosol of human erythrocytes (Figure 3).

In the work presented here, we demonstrate that, in human and rabbit erythrocytes, cGMP can inhibit hydrolysis of cAMP. When rabbit erythrocytes were incubated with dbcGMP, a cell permeable cGMP analog that is converted to cGMP within cells via hydrolyases [40,42–44], cAMP levels were increased in a concentration-dependent manner (Figure 4). In addition, we found that ISO-induced increases in cAMP were augmented by dbcGMP (Figure 6) establishing that cGMP can regulate cAMP increases associated with a discrete signaling pathway in rabbit erythrocytes.

However, a more compelling argument for a physiological role of cGMP in the regulation of cAMP levels in erythrocytes is derived from studies in which endogenous cGMP synthesis is stimulated. In order to examine the effects of cGMP in erythrocytes we activated soluble guanylyl cyclase

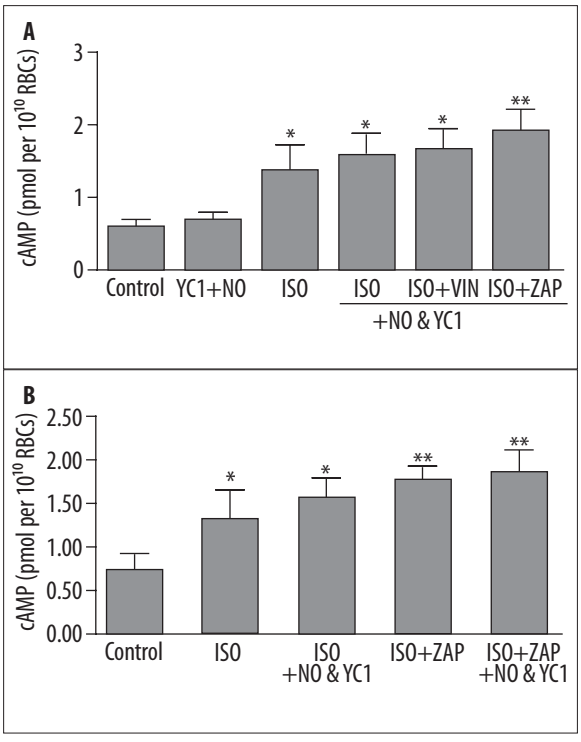

Figure 7. Panel A: Effect of YC1 (100μM) and spermine NONOate (NO, 100 nM) on un-stimulated (control) and isoproterenol (ISO, 1 μM)-induced increases in cAMP in the absence and presence of vinpocetine (VIN, 30 μM) or zaprinast (10 μM) in rabbit erythrocytes (n=5). Erythrocytes were incubated with YC1 and spermine NONOate alone or with VIN or ZAP for 30 min prior to stimulation with ISO for 30 min. Values are means ±SE. * different from control (N',N-dimethylformamide, DMF), ($p<0.01$); ** different from all other values ($P<0.01$). Panel B: Effect of YC1 (100 μM) and spermine NONOate (NO, 100 nM) as well as zaprinast (10 μM) on isoproterenol (ISO, 1 μM)-induced increases in human erythrocytes (n=7). Erythrocytes were incubated with YC1 and spermine NONOate alone, ZAP alone or the combination for 30 min prior to stimulation with ISO for 30 min. Values are means ±SE. * different from control (N',N-dimethylformamide, DMF), ($p<0.01$); ** different from all other values ($P<0.01$).

(sGC) in both rabbit and human erythrocytes with the combination of an NO donor (spermine NONOate, SpNO) and a direct activator of sGC (YC1) in the presence and absence of either a selective inhibitor of PDE1 (VIN) or PDE5 (ZAP).

YC1 is a compound that sensitizes the sGC to NO and enhances the ability of sGC to generate cGMP [24,48,49]. It has also been reported that YC1 can inhibit the activity of some PDEs [49,50]. However, at the concentrations used in our studies, YC1 and SpNO had no effect on cAMP levels in the presence or absence of ISO (Figure 7). In contrast, the selective PDE5 inhibitor, ZAP, significantly increased cAMP levels in erythrocytes in both cases (figure 7). Thus, the effects of YC1 in our studies are not the result of inhibition of PDE activity.

Finally, we exposed erythrocytes to ISO in the absence and presence of either the PDE1 inhibitor, VIN, or the PDE5

inhibitor, ZAP, and measured receptor-mediated cAMP generation [1,10,19,31,35]. The combination of activation of sGC and inhibition of PDE5 potentiated ISO-induced increases in cAMP in both rabbit (Figure 7A) and human erythrocytes (Figure 7B), while inhibition of PDE1 had no effect. These results do not negate the importance of PDE1 in hydrolyzing cGMP in erythrocytes but do support the conclusion that this PDE is not involved in the βAR signaling pathway. Inhibitors of PDE5 are used in the treatment of erectile dysfunction as well as pulmonary hypertension [20,36,51]. These drugs have been associated with decreases in systemic arterial blood pressure, especially when administered in conjunction with nitrates and prostacyclin (PGI_2) analogs presumably due to the increases in cyclic nucleotides leading to vasodilation [52,53]. Although not addressed in the present study, it is possible that a component of the vasodilation associated with the use of PDE5 inhibitors is the result of enhanced cAMP levels in erythrocytes leading to increased ATP release. Such a finding would make the erythrocyte a novel therapeutic target for the development of drugs to lower blood pressure in both the pulmonary and systemic circulations.

Conclusions

We demonstrate, for the first time, the presence of three cytosolic PDEs, PDE4D, PDE3A and PDE5, in human erythrocytes. In addition, we show that cGMP can increase basal cAMP levels and augment increases in cAMP produced by receptor-mediated activation of the βAR in rabbit and human erythrocytes. Finally, we demonstrate that the combination of endogenous activators of sGC (YC1 and SpNO) in combination with a selective inhibitor of PDE5 (zaprinast) augments increases in cAMP produced by ISO. Taken together, these data suggest a heretofore unrecognized role for cGMP and PDE5 in the regulation of agonist-induced increases in cAMP in rabbit and human erythrocytes.

Statement of author contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript.

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